



Scientific Opinion on Flavouring Group Evaluation 302 (FGE.302): N(2-methylcyclohexyl)2,3,4,5,6pentafluorobenzamide from Chemical Group 30

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)

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Scientific Opinion on Flavouring Group Evaluation 302 (FGE.302): *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluorobenzamide from Chemical Group 30

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Abstract

The EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids was requested to evaluate *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluorobenzamide [FL-no: 16.119] in the Flavouring Group Evaluation 302, using the Procedure in Commission Regulation (EC) No 1565/2000. The substance is intended to be used as a flavour modifier and the current evaluation is only applicable to this use. Information on the ratios of diastereoisomers of the substance has been provided (*cis* 20–40% and *trans* 60–80%). Information on the ratio of enantiomers is lacking. The available data on genotoxicity do not preclude the evaluation of the candidate substance [FL-no: 16.119] through the Procedure. The substance was evaluated through the B-side of the Procedure. A 'No Observed Adverse Effect Level' (NOAEL) of 55 mg/kg body weight (bw) per day could be derived for [FL-no: 16.119] from a 90-day subchronic toxicity study in rats. This NOAEL provides an adequate margin of safety of 1.4×10^6 , based on the 'Maximised Survey-Derived Daily Intake' (MSDI) of 2.4 µg/capita per day. Based on the 'modified Theoretical Added Maximum Daily Intake' (mTAMDI) approach, the Panel concluded that more information is needed on use and use levels. Besides the safety assessment of this flavouring substance, the specifications for the material of commerce have also been considered. Additional information on the stereoisomeric composition of the flavouring substance is required.

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Requestor: European Commission

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The use of flavourings in food is regulated under Regulation (EC) No 1334/2008 of the European Parliament and Council of 16 December 2008¹ on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union list of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012². The list includes flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000³.

During its 16th plenary meeting of 28–30 September 2010, the EFSA Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) decided to put the evaluation of the flavouring substance *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] from Flavouring Group Evaluation 302 (FGE.302) on hold as the data available were not considered to be sufficient for an evaluation: no adequate toxicity study was available, from which a No Observed Adverse Effect Level (NOAEL) could be established, neither for the substance itself nor for any structurally related substance.

The Panel concluded that the safety of the substance, when used as a flavouring substance, could therefore not be assessed and requested additional data, which would include absorption, distribution, metabolism, elimination (ADME) data and an extended 90-day study, including endpoints on reprotoxicity.

On 18 November 2013, the applicant submitted data on *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119].

Terms of Reference as provided by the European Commission

The European Commission requests the European Food Safety Authority (EFSA) to finalise its safety assessment on this flavouring substance in accordance with Commission Regulation (EC) No 1565/2000.

Interpretation of the Terms of Reference

The present scientific opinion FGE.302 covers the safety assessment of the following flavouring substance: *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL 16.119]. This substance will be evaluated as a flavour-modifying substance (cf. Regulation (EC) No 1334/2008 for concentrations up to 5 mg/kg in solution (Dewis et al., 2009). Although [FL-no: 16.119] has also been reported to be added to food as a sweetener and flavour enhancer⁴ (Dewis et al., 2009), these uses are out of the scope of the present evaluation which only assess the use of the substance as a flavour modifier. Use as a sweetener or a flavour enhancer would fall under the food additive legislation⁵ which has different data requirements for safety evaluation.

2. Assessment

2.1. Presentation of the substances in Flavouring Group Evaluation 302

2.1.1. Description

The present FGE.302, using the procedure as referred to in the Commission Regulation (EC) No 1565/2000 (The Procedure – shown in schematic form in Appendix A), deals with the safety evaluation of the flavouring substance *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] from chemical group 30, Annex I of Commission Regulation (EC) No 1565/2000³. The

¹ Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34–50.

² Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1–161.

³ Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8–16.

⁴ <http://www.thegoodscentscompany.com/data/rw1119821.html>

⁵ Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.

flavouring substance (candidate substance) under consideration, as well as the chemical name, FLAVIS- (FL-), Chemical Abstract Service- (CAS-), Council of Europe- (CoE-) and Flavor and Extract Manufacturers Association- (FEMA-) numbers, structure and specifications, are listed in Table 1.

N-(2-Methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as of no safety concern (JECFA, 2012). However, this evaluation was based on a NOAEL issued from a 28-day study in rats. Currently, a 90-day study has become available and evaluated in the present FGE.

According to publically available information⁴ and Dewis et al. (2009), [FL-no: 16.119] can be used as a sweetener or as a flavour enhancer. In addition, the substance can also be used as a flavour modifier at concentrations up to 5 mg/kg in solutions. This opinion concerns only the safety of the latter use.

No sufficiently structurally related substances were identified.

2.1.2. Stereoisomers

It is recognised that geometrical and optical isomers of substances may have different properties. Their flavour may be different; they may have different chemical properties resulting in possible variability in their absorption, distribution, metabolism, elimination and toxicity. Thus, information must be provided on the configuration of the flavouring substance, i.e. whether it is one of the geometrical/optical isomers, or a defined mixture of stereoisomers. The available specifications of purity will be considered in order to determine whether the safety evaluation carried out for a candidate substance for which stereoisomers may exist, can be applied to the material of commerce. Flavouring substances with different configurations should have individual chemical names and codes (CAS number, FLAVIS number, etc.).

The candidate substance belongs to structural class III. It possesses two chiral centres. Information on the ratios of the diastereoisomers has been provided (*cis* 20–40% and *trans* 60–80%) (Flavour Industry, 2015). Information on the ratio of enantiomers is lacking.

2.1.3. Natural occurrence in food

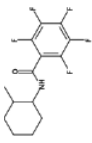
The candidate substance [FL-no: 16.119] has not been reported to occur naturally in food (Triskelion, 2016).

2.2. Specifications

Purity criteria for the candidate substance *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] have been provided by the flavour industry (Flavour Industry, 2009, 2015) (Table 1).

Judged against the requirements in Annex II of Commission Regulation (EC) No 1565/2000³, information on the ratios of the diastereoisomers has been provided (*cis* 20–40% and *trans* 60–80%) (Flavour Industry, 2015). Information on the ratio of enantiomers is lacking.

Table 1: Specification summary of the candidate substance in the Flavouring Group Evaluation 302

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility ^(a) Solubility in ethanol ^(b)	Boiling point, °C ^(c) Melting point, °C ID test Assay minimum	Refrac. index ^(d) Spec. gravity ^(e)	Specification comments
16.119 2081	N-(2-Methylcyclohexyl)-2,3,4,5, 6-pentafluoro-benzamide		4678 1003050-32-5	Solid C ₁₄ H ₁₄ F ₅ NO 307.10	Slightly soluble Soluble	n.a. 150–152 IR NMR MS 98%	n.a. 1.12	The stereoisomeric composition is given as a ratio of <i>cis</i> to <i>trans</i> isomer (<i>cis</i> 20–40% and <i>trans</i> 60–80%) (Flavour Industry, 2015) Information on the ratio of enantiomers is lacking

FL-no: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; ID: identity; IR: infrared; NMR: nuclear magnetic resonance; MS: mass spectrometry.
n.a.: not applicable.

(a): Solubility in water, if not otherwise stated.

(b): Solubility in 95% ethanol, if not otherwise stated.

(c): At 1013.25 hPa (1 atm), if not otherwise stated.

(d): At 20°C, if not otherwise stated.

(e): At 25°C, if not otherwise stated.

2.3. Intake data

Annual production volumes of the flavouring substance as surveyed by the flavour industry can be used to calculate the 'Maximised Survey-derived Daily Intake' (MSDI) by assuming that the production figure only represents 60% of the use in food due to underreporting and that 10% of the total European Union (EU) population are consumers (SCF, 1999).

However, the Panel noted that due to year-to-year variability in production volumes, to uncertainties in the underreporting correction factor and to uncertainties in the percentage of consumers, the reliability of intake estimates on the basis of the MSDI approach is difficult to assess.

The Panel also noted that in contrast to the generally low per capita intake figures estimated on the basis of this MSDI approach, in some cases the regular consumption of products flavoured at use levels reported by the flavour industry in the submissions would result in much higher intakes. In such cases, the human exposure thresholds below which exposures are not considered to present a safety concern might be exceeded.

Considering that the MSDI model may underestimate the intake of a flavouring substance by certain groups of consumers, the Scientific Committee on Food (SCF) recommended also taking into account the results of other intake assessments (SCF, 1999).

One of the alternatives is the 'Theoretical Added Maximum Daily Intake' (TAMDI) approach, which is calculated on the basis of standard portions and upper use levels (SCF, 1995) for flavourable beverages and foods in general, with exceptional levels for particular foods. This method is regarded as a conservative estimate of the actual intake by most consumers because it is based on the assumption that the consumer regularly eats and drinks several food products containing the same flavouring substance at the upper use level.

One option to modify the TAMDI approach is to base the calculation on normal rather than upper use levels of the flavouring substance. This modified approach is less conservative (e.g. it may underestimate the intake of consumers being loyal to products flavoured at the maximum use levels reported). However, it is considered as a suitable tool to screen and prioritise the flavouring substances according to the need for refined intake data (EFSA, 2004).

2.3.1. Estimated daily per capita intake (MSDI approach)

The intake estimation is based on the MSDI approach, which involves the acquisition of data on the amounts used in food as flavourings (SCF, 1999). These data are derived from surveys on annual production volumes in Europe. These surveys were conducted in 1995 by the International Organization of the flavour industry (IOFI), in which flavour manufacturers reported the total amount of each flavouring substance incorporated into food sold in the EU during the previous year (IOFI, 1995). The intake approach does not consider the possible natural occurrence in food.

Average per capita intake (MSDI) is estimated on the assumption that the amount added to food is consumed by 10% of the population⁶ (Eurostat, 1998). This is derived for candidate substances from estimates of annual volume of production provided by the flavour industry and incorporates a correction factor of 0.6 to allow for incomplete reporting (60%) in the flavour industry surveys (SCF, 1999).

The anticipated total annual volume of production of the candidate substance in the present Flavouring Group Evaluation (FGE.302) from use as flavouring substance in Europe has been reported to be approximately 20 kg (Flavour Industry, 2009). The daily per capita intake for the substance is 2.4 µg/capita per day (Table 3).

2.3.2. Intake estimated on the basis of the modified TAMDI (mTAMDI)

The method for calculation of modified TAMDI (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995).

The assumption is that a person may consume a certain amount of flavourable foods and beverages per day.

⁶ EU figures 375 million. This figure relates to EU population at the time for which production data are available, and is consistent (comparable) with evaluations conducted prior to the enlargement of the EU. No production data are available for the enlarged EU (Eurostat, 1998).

For the candidate substance information on food categories and normal and maximum use levels^{7,8} were submitted by the flavour industry (Flavour Industry, 2014). The candidate substance is used in flavoured food products divided into the food categories, outlined in Annex III of the Commission Regulation (EC) No 1565/2000³, as shown in Table 2. For the present calculation of mTAMDI, the reported normal use levels were used. In the case where different use levels were reported for different food categories, the highest reported normal use level was used.

Table 2: Use of the candidate substance

Food category	Description	Flavouring used
01.0	Dairy products, excluding products of category 2	Yes
02.0	Fats and oils, and fat emulsions (type water-in-oil)	No
03.0	Edible ices, including sherbet and sorbet	Yes
04.1	Processed fruits	Yes
04.2	Processed vegetables (including mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	Yes
05.0	Confectionery	Yes
06.0	Cereals and cereal products, including flours & starches from roots & tubers, pulses & legumes, excluding bakery	No
07.0	Bakery wares	Yes
08.0	Meat and meat products, including poultry and game	No
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	No
10.0	Eggs and egg products	No
11.0	Sweeteners, including honey	No
12.0	Salts, spices, soups, sauces, salads, protein products etc.	No
13.0	Foodstuffs intended for particular nutritional uses	No
14.1	Non-alcoholic ('soft') beverages, excluding dairy products	Yes
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts	No
15.0	Ready-to-eat savouries	No
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 1–15	No

According to the flavour industry, the normal use levels for the candidate substance are in the range of 1–5 mg/kg food, and the maximum use levels are in the range of 2–25 mg/kg (Flavour Industry, 2014).

The mTAMDI value is 520 µg/person per day for the candidate substance from structural class III (see Section 6).

For detailed information on use levels and intake estimations based on the mTAMDI approach, see Appendix C.

3. Absorption, distribution, metabolism and elimination

The kinetics of the candidate substance have been investigated in microsomes, in hepatocytes and *in vivo* by autoradiography (¹⁴C incorporated on the carbonyl group) after a single oral administration to rats.

Results from the *in vitro* study on metabolism in rat liver microsomes show that ¹⁴C radiolabelled *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide is readily hydroxylated in liver microsomes incubated with NADPH generating system as a co-factor. These metabolites may be anticipated to be easily conjugated and excreted.

Also, in suspension cultures of cryopreserved hepatocytes incubated with [FL-no: 16.119], rapid metabolism of the candidate flavouring substance was observed but no attempt was made to identify

⁷ 'Normal use' is defined as the average of reported usages and 'maximum use' is defined as the 95th percentile of reported usages (EFFA, 2002).

⁸ The normal and maximum use levels in different food categories have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004).

the biotransformation products. Next to hydroxylation, hydrolysis of the amide bond might occur which would result in formation of 2-methyl-cyclohexylamine and pentafluorobenzoic acid. However, there are no experimental data to support this. In addition, there is no information whether defluorination *in vivo* might occur but from the molecular structure this seems unlikely.

In the autoradiography study, there was still radioactivity in tissues 72 hours after dosage but the studies do not suggest retention in a specific organ and indicate an efficient elimination within the observation period.

Some perfluorinated alkyl substances are bioaccumulative (EFSA, 2008; EFSA CEF Panel, 2010). Although the candidate substance is not a perfluorinated alkyl substance, the presence of a perfluorinated aryl moiety might suggest a potential bioaccumulation. However, the above described experimental data indicate that for *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide bioaccumulation is not of concern.

From the available information, it is not possible to conclude how the candidate substance will be metabolised *in vivo*. Therefore, it cannot be anticipated that the candidate substance [FL-no: 16.119] will be metabolised to innocuous products.

For more detailed information, see Appendix D.

4. Application of the Procedure for the safety evaluation of flavouring substances

The application of the Procedure is based on intakes estimated on the basis of the MSDI approach. Where the mTAMDI approach indicates that the intake of a flavouring substance might exceed its corresponding threshold of concern, a formal safety assessment is not carried out using the Procedure. In these cases, the Panel requires more precise data on use and use levels. For comparison of the intake estimations based on the MSDI approach and the mTAMDI approach, see Section 5.

For the safety evaluation of the candidate substance from chemical group 30, the Procedure as outlined in Appendix A was applied, based on the MSDI approach. The stepwise evaluation of the substance is summarised on Figure A.1 and Table B.1.

Step 1

The candidate substance *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] is classified into structural class III according to the decision tree approach by Cramer et al. (1978).

Step 2

Step 2 requires consideration of the metabolism of the candidate substances. The candidate substance [FL-no: 16.119] cannot be anticipated to be metabolised to innocuous products and thus the evaluation proceeds via the B-side of the Procedure scheme.

Step B3

The estimated daily per capita intake of the candidate substances [FL-no: 16.119] is 2.4 µg, which is below the threshold for its structural class of 90 µg/person per day (class III). Accordingly, the evaluation of the substance proceeds to step B4 of the Procedure.

Step B4

A NOAEL of 55 mg/kg bw per day may be derived from a 90-day study providing an adequate margin of safety of 1.4×10^6 . Thus, it can be concluded that *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] is not of safety concern at the estimated levels of intake based on the MSDI approach.

5. Comparison of the intake estimations based on the MSDI approach and the mTAMDI approach

The estimated intake of the candidate substance [FL-no: 16.119] assigned to structural class III, based on the mTAMDI, is 520 µg/person per day, which is above the threshold of concern for structural class III of 90 µg/person per day.

Thus, for the candidate substances [FL-no: 16.119], further information is required. This would include more reliable intake data and then, if required, additional toxicological data.

For comparison of the intake estimates based on the MSDI approach and the mTAMDI approach, see Table 3.

Table 3: Estimated intakes based on the MSDI approach and the mTAMDI approach

FL-no	EU Register name	MSDI (µg/capita per day)	mTAMDI (µg/person per day)	Structural class	Threshold of concern (µg/person per day)
16.119	<i>N</i> -(2-Methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide	2.4	520	Class III	90

MSDI: Maximised Survey-derived Daily Intake; mTAMDI: modified Theoretical Added Maximum Daily Intake.

6. Considerations of combined intakes from use as flavouring substances

Combined intake is not relevant as this FGE concerns only one candidate substance and no structurally related substances were available.

7. Toxicity

7.1. Acute toxicity

No data on acute toxicity have been submitted, and no data on acute toxicity of the candidate substance [FL-no: 16.119] were found when a literature search was conducted.

7.2. Subacute, subchronic, chronic and carcinogenicity studies

***N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119]**

28-day feeding toxicity study in rats

A 28-day feeding study in rats was conducted for the candidate substance *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] at doses of 0, 750, 1,500 and 3,000 mg/kg which correspond to dose levels of 0, 67, 130 and 262 mg/kg bw per day, respectively. The control group and each dosage group consisted of five female and five male rats.

In all dosage groups, reduction in mobile activity was noted; however, evidence of neurotoxicity was absent. A reduction in body weight gain was observed in the highest dose group, which was statistically significant for males but not for females. In the high-dose animals, a reduction in food efficiency was noted during part of the study time. Males in the highest dose group showed reduction in red blood cell count, increases in neutrophil counts and increase in alkaline phosphatase, which were statistically significant. Females in the highest dose group demonstrated statistically significant increases in total protein, albumin and total cholesterol; they also showed reduction in absolute spleen weights and increase in relative spleen weights. In males from the two highest dosage groups, increase in activated partial thromboplastin time was observed. In all female treatment groups, a statistically significant increase in absolute and relative liver weights were noted, but without dose-response. No histopathological changes were observed (Dunster, 2009).

90-day subchronic toxicity study in rats

A 90-day dietary study was performed with *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] (Bauter, 2013) according to OECD guideline (TG 408) and the requirements of US FDA Good Laboratory Practice (GLP) Standards.

Four groups (10/sex per dietary intake level) of male and female CRL Wistar Han IGS rats were fed a diet that contained 0 (dietary control), 750 (low-dose group), 1,500 (mid-dose group) or 3,000 (high-dose group) mg/kg *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide. The levels corresponded to a calculated daily intake of 0, 55, 108 and 218 mg/kg bw, respectively, for males and 0, 66, 125 or 255 mg/kg bw, respectively, for females. Stability of the test substance under storage conditions as well as the stability of the test substance in the feed matrix was found to be adequate. Blood samples for haematology and clinical chemistry analysis were drawn on days 43 and 85 of the study. Urine samples were also collected on days 43 and 85. All animals survived the course of the study, and were sacrificed on day 93. Material from ca 40 tissues/locations was collected and preserved for all animals. However, only tissues from the control and high-dose groups were fully examined. For the low- and mid-dose groups only tissues for which in the high-dose group an effect

was observed, tissues with gross lesions and all sex organs were analysed. A neurobehavioural functional observational battery was not included in the study design.

Analysis of plasma was made for the parent compound and a putative hydroxymetabolite was performed for all groups. Trace levels of the parent and/or putative hydroxymetabolite were detected in all three groups receiving the test substance. Limit of quantification was 1 ng/mL (ppb). Measured concentrations ranged from 1 to 4.94 ng/mL, with the highest value for the putative metabolite in the plasma of an animal in the high-dose group.

Clinical signs attributed to the test substance were a dietary concentration dependent increase in severity of whitening of the teeth, which was noted for the mid- and high-dose groups, beginning on day 36 and continuing throughout the course of the study. Three high-dose group males had broken upper incisor teeth days 42–93, which was potentially attributed to the observed enamel degeneration. Clinical examination did not reveal indications of behavioural changes (e.g. changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements), stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards).

Decreased body weight and body weight gain in the high-dose group males and females were attributed to decreased food intake, related to the test substance at high levels and considered non-adverse.

Statistically significant differences ($p < 0.05$) in haematology parameters for male rats included decreased monocyte concentration in the mid-dose group on day 43, and decreased neutrophil and eosinophil concentration in the mid-dose group on day 85 and increased large unstained cell concentration in the high-dose group on day 85. Female rats showed statistically significant ($p < 0.05$) decreased red blood cell concentration in the high-dose group on day 43, and decreased haemoglobin and haematocrit in the mid- and high-dose groups on days 43 and 85.

Statistically significant differences ($p < 0.05$) that were measured for clinical chemistry parameters included dose-dependent decreased serum alanine aminotransferase which was significant in high-dose males at days 43 and 85 and in high-dose females at day 43. Male rats in the high-dose group had significantly decreased sorbitol dehydrogenase levels days 43 and 85, the same dose-dependent tendency was found in females although not significant. Blood glucose showed a dose-dependent decrease and was significantly lowered in high-dose males at day 85 and in mid- and high-dose females on day 85. A dose-dependent increase in serum inorganic phosphorous levels was apparent in both male and female rats; significantly increased levels were measured in high-dose males on day 43 and in both mid- and high-dose males on day 85.

Analysis of urine showed significantly increased urine volume for males in the high-dose group at day 85, and reduced specific gravity and reduced urinary protein at days 43 and 85 ($p < 0.05$ except for urinary protein day 85 where $p < 0.002$). For these three parameters, the changes were dose-dependent and were apparent also in females, although not statistically significant.

Histopathology findings of note were degeneration of teeth in the nose/nasal turbinate sections of all animals, males and females, in the high-dose group. Findings included disorganisation of the ameloblast layer, accompanied by irregular deposits of enamel and less dentin in each affected tooth. The teeth were misshaped. These findings correspond to the concentration-dependent whitening of the teeth in the mid- and high-dose groups that were apparent from day 36 and onwards, and also with the broken upper incisor teeth during the latter part of the study that occurred in three males in the high-dose group. No alterations were observed in the rats molar teeth. However, the Panel noted that the use of the rat model with respect to this type of dental issue is due to the fast growth of the incisors and such a growth would not be expected to occur in the molars. There were no changes in the bones (femur) from control and high-dose males and females.

The effect on teeth observed both by clinical inspection and as histopathology findings gives a strong suspicion of fluorosis. The aspect of fluorosis has, however, not been discussed in the study report. ADME data submitted by the applicant indicate that *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluorobenzamide [FL-no: 16.119] is not extensively hydrolysed by liver microsomes *in vitro*. The only metabolites that have been identified were those where the parent compound had been hydroxylated in different positions on the cyclohexane ring (see Section 4 and Appendix D). Upon request further information on stability of the *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluorobenzamide molecule [FL-no: 16.119] has been provided by industry, as well as results from analysis of the test substance and the feed for free fluoride (Flavouring Industry, 2015). Results indicate that *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluorobenzamide [FL-no: 16.119] is stable over time. Analysis of three manufactured batches of the test substance showed some variation in fluoride ion concentration between samples of the same lot, but the average results were similar from lot-to-lot and the average detectable level of

fluoride ions was 0.7 mg/kg. No detectable amounts of fluoride were found above the limit of determination when the rat feed was analysed; results are reported as < 0.3 mg/kg free fluoride ion in feed. If the effect on teeth in the 90-day study is indeed fluorosis, the source of fluoride is not known. The rat, in particular, has proved to be a good model for human dental fluorosis, as the rat shows the same disturbances at similar plasma levels as humans. However, rats require approximately 10 times higher dosages of fluoride than humans to achieve the same plasma levels. In general, fluoride doses that induce cellular changes in ameloblasts are at least two orders of magnitude higher than those that induce mineralisation disturbances (Bronckers et al., 2009; Perumal et al., 2013).

Based on clinical observations of teeth, histopathology observations on ameloblasts and enamel and dentin in teeth and results from clinical chemistry analysis, the NOAEL for *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] that may be derived from the above detailed 90-day study in rats is 750 mg/kg feed, corresponding to an estimated daily intake of 55 and 66 mg/kg bw per day for males and females, respectively.

Repeated dose toxicity data are summarised in Table E.3 (Appendix E).

7.3. Developmental/reproductive toxicity studies

There are no developmental/reproductive toxicity studies for the candidate substance. The 90-day study described in Section 7.2 included investigations of sperm motility, epididymal sperm count, homogenisation-resistant spermatid count and sperm morphological development, no effects attributable to the test substance were found. Similarly, there were no changes in mean oestrus cycle length, number of oestrus cycles or any microscopic changes in uterus, vagina, ovaries, oviducts or cervix in treated females (Bauter, 2013).

7.4. Genotoxicity

The candidate substance, *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] is reported to be negative in two Ames tests, with and without metabolic activation (Kirby, 2008).

In an *in vivo* study, *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] (purity > 98%) was reported negative in a micronucleus test in mouse bone marrow (Song, 2008). The flavouring substance and vehicle control were administered by oral gavage. Three dose levels were used in the assay: 500, 1,000 and 2,000 mg/kg bw. Dimethyl sulfoxide (DMSO) was used as a vehicle control and a single, oral, gavage dose of cyclophosphamide of 80 mg/kg bw as a positive control. Animals were killed 24 and 48 h after dosing. A total of 2,000 polychromatic erythrocytes (PCE) per animal were analysed for frequency of micronuclei. No reduction (more than 20% vs that of the vehicle control) in the percentage of PCE was observed, in all but one treatment group. The decrease in PCE/normochromatic erythrocytes (NCE) ratio by 21.1% in male mice at 1,000 mg/kg (48 h) appears an isolated observation and not related to treatment. An autoradiography study performed in rats showed that after a single oral dose treatment with [¹⁴C]-*N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide to rats, radioactivity was widely distributed in all tissues supporting systemic exposure (Appendix E).

Conclusion on genotoxicity:

Data on genotoxicity do not preclude the evaluation of the candidate substance [FL-no: 16.119] through the Procedure.

Genotoxicity data are summarised in Tables E.1 and E.2 (Appendix E).

8. Conclusion

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the CEF Panel) was asked to deliver a scientific opinion on the implications for human health of a chemically defined flavouring substance used in or on foodstuffs in the Member States. In particular, the Panel was requested to evaluate the flavouring substance *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] in the FGE.302, using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000. This flavouring substance belongs to chemical group 30, Annex I of the Commission Regulation (EC) No 1565/2000³. The flavouring substance is listed in the Union List, which was adopted by Commission Regulation (EU) No 872/2012² and its consecutive amendments. The current evaluation is applicable only for the use of this substance as a flavour modifier.

The candidate substance belongs to structural class III and it has not been reported to occur naturally in food. It possesses two chiral centres. Information on the ratios of the diastereoisomers has been provided (*cis* 20–40% and *trans* 60–80%). Information on the absolute configurations at the two asymmetric centres is lacking.

Data on genotoxicity do not preclude the evaluation of the candidate substance [FL-no: 16.119] through the Procedure.

According to the default MSDI approach, the candidate substance has an intake in Europe of 2.4 µg/capita per day, which is below the threshold of concern value for structural class III (90 µg/person per day).

The results of studies on metabolism and pharmacokinetics do not allow to conclude that the metabolites of the candidate substance are innocuous. Accordingly, the [FL-no: 16.119] is evaluated via the B-side of the Procedure scheme.

A NOAEL of 55 mg/kg bw per day may be derived from a 90-day oral toxicity study providing an adequate margin of safety of 1.4×10^6 . Thus, it can be concluded that *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] is not of safety concern at the estimated levels of intake based on the MSDI approach.

In order to determine whether the conclusion for the candidate substance evaluated through the Procedure can be applied to the material of commerce, it is necessary to consider the available specifications. For the material of commerce, information on the proportion of enantiomers has to be provided.

When the estimated intake was based on the mTAMDI, it amounted to 520 µg/person per day for the candidate substance [FL-no: 16.119]. This intake is above the threshold of concern value for the structural class III (90 µg/person per day). Therefore, according to standard practice used by the Panel, more specific information on uses and use levels is required for the candidate substance.

The Panel noted that the candidate substance is polyfluorinated and therefore the substance or breakdown products may be persistent in the environment.

Documentation provided to EFSA

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- 3) EFFA (European Flavour and Fragrance Association), 2002. Letter from EFFA to Dr. Joern Gry, Danish Veterinary and Food Administration. Dated 31 October 2002. Re.: Second group of questions. FLAVIS/8.26.
- 4) EFFA (European Flavour and Fragrance Association), 2004. Intake – Collection and collation of usage data for flavouring substances. Letter from Dan Dils, EFFA to Torben Hallas-Møller, EFSA. May 31, 2004.
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- 6) Flavour Industry, 2014. Unpublished information submitted by the flavour industry to EFSA and forwarded to FLAVIS Secretariat. A-302 [FL-no: 16.119].
- 7) Flavour Industry, 2015. Unpublished information submitted by the flavour industry to EFSA and forwarded to FLAVIS Secretariat. A-302 [FL-no: 16.119].
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Abbreviations

ADME	absorption, distribution, metabolism, elimination
CAS	Chemical Abstract Service
CEF	EFSA Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
DMSO	dimethyl sulfoxide
EFFA	European Flavour and Fragrance Association
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
HPLC	high-performance liquid chromatography
ID	identity
IOFI	International Organization of the Flavour Industry
IR	infrared spectroscopy
IS	internal standard
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LC-MS/MS	liquid chromatography–tandem mass spectrometry
MS	mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	modified Theoretical Added Maximum Daily Intake
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NCE	normochromatic erythrocytes
NMR	nuclear magnetic resonance
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
PCE	polychromatic erythrocytes
SCF	Scientific Committee on Food
TAMDI	Theoretical Added Maximum Daily Intake
US FDA	US Food and Drug Administration
WHO	World Health Organization

Appendix A – Procedure for the safety evaluation

The approach for a safety evaluation of chemically defined flavouring substances as referred to in Commission Regulation (EC) No 1565/2000³, named the 'Procedure', is shown in schematic form in Figure A.1. The Procedure is based on the Opinion of the Scientific Committee on Food expressed on 2 December 1999 (SCF, 1999), which is derived from the evaluation Procedure developed by the Joint FAO/WHO Expert Committee on Food Additives at its 44th, 46th and 49th meetings (JECFA, 1995, 1996, 1997, 1999).

The Procedure is a stepwise approach that integrates information on intake from current uses, structure–activity relationships, metabolism and, when needed, toxicity. One of the key elements in the Procedure is the subdivision of flavourings into three structural classes (I, II and III) for which thresholds of concern (human exposure thresholds) have been specified. Exposures below these thresholds are not considered to present a safety concern.

Class I contains flavourings that have simple chemical structures and efficient modes of metabolism, which would suggest a low order of oral toxicity. Class II contains flavourings that have structural features that are less innocuous, but are not suggestive of toxicity. Class III comprises flavourings that have structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity (Cramer et al., 1978). The thresholds of concern for these structural classes of 1,800, 540 or 90 µg/person per day, respectively, are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996).

In Step 1 of the Procedure, the flavourings are assigned to one of the structural classes. The further steps address the following questions:

- Can the flavourings be predicted to be metabolised to innocuous products⁹ (Step 2)?
- Do their exposures exceed the threshold of concern for the structural class (Steps A3 and B3)?
- Are the flavourings or their metabolites endogenous¹⁰ (Step A4)?
- Does a NOAEL exist on the flavourings or on structurally related substances (Steps A5 and B4)?

In addition to the data provided for the flavouring substances to be evaluated (candidate substances), toxicological background information available for compounds structurally related to the candidate substances is considered (supporting substances), in order to assure that these data are consistent with the results obtained after application of the Procedure.

The Procedure is not to be applied to flavourings with existing unresolved problems of toxicity. Therefore, the right is reserved to use alternative approaches if data on specific flavourings warranted such actions.

⁹ 'Innocuous metabolic products': Products that are known or readily predicted to be harmless to humans at the estimated intakes of the flavouring agent (JECFA, 1997).

¹⁰ 'Endogenous substances': Intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included (JECFA, 1997).

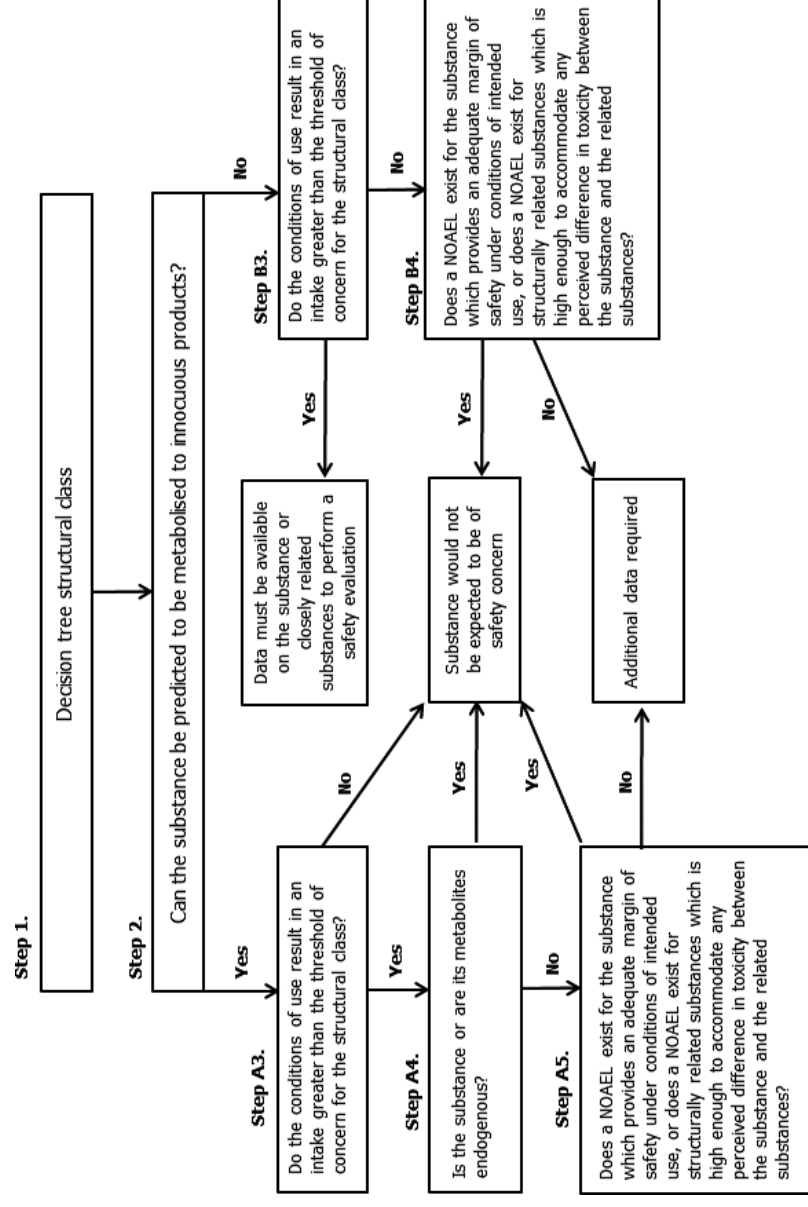
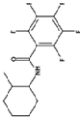


Figure A.1: Procedure for safety evaluation of chemically defined flavouring substances

Appendix B – Summary of the safety evaluation

Table B.1: Summary of the safety evaluation applying the Procedure

FL-no	EU Register name	Structural formula	MSDI ^(a) (µg/capita per day)	Class ^(b) Evaluation procedure path ^(c)	Outcome on the named compound ^(d) or ^(e)	Outcome on the material of commerce ^(f) , ^(g) or ^(h)
16.119 2081	<i>N</i> -(2-Methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide		2.4	Class III B4: adequate NOAEL exists	d	h

MSDI: Maximised Survey-derived Daily Intake; NOAEL: No-Observed-Adverse-Effect-Level.

(a): EU MSDI: Amount added to food as flavour in (kg/year) \times 10E9/(0.1 \times population in Europe (= 375 \times 10E6) \times 0.6 \times 365) = µg/capita per day.

(b): Thresholds of concern: Class I = 1,800 µg/person per day; Class II = 540 µg/person per day; Class III = 90 µg/person per day.

(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot be predicted to be metabolised to innocuous products.

(d): No safety concern based on intake calculated by the MSDI approach of the named compound.

(e): Data must be available on the substance or closely related substances to perform a safety evaluation.

(f): No safety concern at the estimated level of intake of the material of commerce meeting the specification requirement (based on intake calculated by the MSDI approach).

(g): Tentatively regarded as presenting no safety concern (based on intake calculated by the MSDI approach) pending further information on the purity of the material of commerce and/or information on stereoisomerism.

(h): No conclusion can be drawn due to lack of information on the purity of the material of commerce.

Appendix C – Use levels/mTAMDI

C.1. Normal and maximum use levels

For each of the 18 food categories (Table C.1) in which the candidate substances are used, flavour industry reports a 'normal use level' and a 'maximum use level'.³ According to the flavour industry, the 'normal use' is defined as the average of reported usages and 'maximum use' is defined as the 95th percentile of reported usages (EFFA, 2002). The normal and maximum use levels in different food categories have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004).

Table C.1: Food categories according to Commission Regulation (EC) No 1565/2000³

Food category	Description
01.0	Dairy products, excluding products of category 02.0
02.0	Fats and oils, and fat emulsions (type water-in-oil)
03.0	Edible ices, including sherbet and sorbet
04.1	Processed fruit
04.2	Processed vegetables (including mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds
05.0	Confectionery
06.0	Cereals and cereal products, including flours & starches from roots & tubers, pulses & legumes, excluding bakery
07.0	Bakery wares
08.0	Meat and meat products, including poultry and game
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms
10.0	Eggs and egg products
11.0	Sweeteners, including honey
12.0	Salts, spices, soups, sauces, salads, protein products, etc.
13.0	Foodstuffs intended for particular nutritional uses
14.1	Non-alcoholic ('soft') beverages, excluding dairy products
14.2	Alcoholic beverages, including alcohol-free and low-alcoholic counterparts
15.0	Ready-to-eat savouries
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0

The 'normal and maximum use levels' for the candidate substance are reported in Table C.3.

C.2. mTAMDI calculations

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995). The assumption is that a person may consume the amount of flavourable foods and beverages listed in Table C.2. These consumption estimates are then multiplied by the reported use levels in the different food categories and summed up.

Table C.2: Estimated amount of flavourable foods, beverages, and exceptions assumed to be consumed per person per day (SCF, 1995)

Class of product category	Intake estimate (g/day)
Beverages (non-alcoholic)	324.0
Foods	133.4
Exception a: Candy, confectionery	27.0
Exception b: Condiments, seasonings	20.0
Exception c: Alcoholic beverages	20.0
Exception d: Soups, savouries	20.0
Exception e: Others, e.g. chewing gum	e.g. 2.0 (chewing gum)

The mTAMDI calculations are based on the normal use levels reported by industry. The seven food categories used in the SCF TAMDI approach (SCF, 1995) correspond to the 18 food categories as outlined in Commission Regulation (EC) No 1565/2000³ and reported by the flavour industry in the following way (see Table C.4):

- Beverages (SCF, 1995) correspond to food category 14.1
- Foods (SCF, 1995) correspond to the food categories 1, 2, 3, 4.1, 4.2, 6, 7, 8, 9, 10, 13 and/or 16
- Exception a (SCF, 1995) corresponds to food category 5 and 11
- Exception b (SCF, 1995) corresponds to food category 15
- Exception c (SCF, 1995) corresponds to food category 14.2
- Exception d (SCF, 1995) corresponds to food category 12
- Exception e (SCF, 1995) corresponds to others, e.g. chewing gum.

Table C.3: Normal and maximum use levels (mg/kg) for the candidate substance in FGE. 302 (Flavour Industry, 2014)

FL-no	Food categories																	
	Normal use levels (mg/kg)																	
	Maximum use levels (mg/kg)																	
	01.0	02.0	03.0	04.1	04.2	05.0	06.0	07.0	08.0	09.0	10.0	11.0	12.0	13.0	14.1	14.2	15.0	16.0
16.119	1	–	1	1	1	–	1	1	–	–	–	1	–	1	1	–	–	–
	5	–	5	5	5	–	5	2	–	–	–	25	–	5	3	–	–	–

Table C.4: Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000³ into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

Food categories according to Commission Regulation (EC) No 1565/2000			Distribution of the seven SCF food categories		
Key	Food category		Foods	Beverages	Exceptions
01.0	Dairy products, excluding products of category 02.0		Foods		
02.0	Fats and oils, and fat emulsions (type water-in-oil)		Foods		
03.0	Edible ices, including sherbet and sorbet		Foods		
04.1	Processed fruit		Foods		
04.2	Processed vegetables (including mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds		Foods		
05.0	Confectionery				Exception a
06.0	Cereals and cereal products, including flours & starches from roots & tubers, pulses & legumes, excluding bakery		Foods		
07.0	Bakery wares		Foods		
08.0	Meat and meat products, including poultry and game		Foods		
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms		Foods		
10.0	Eggs and egg products		Foods		
11.0	Sweeteners, including honey				Exception a
12.0	Salts, spices, soups, sauces, salads, protein products, etc.				Exception d
13.0	Foodstuffs intended for particular nutritional uses		Foods		
14.1	Non-alcoholic ('soft') beverages, excluding dairy products			Beverages	
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts				Exception c
15.0	Ready-to-eat savouries				Exception b
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0		Foods		

The mTAMDI value (see Table C.5) is presented for the flavouring substance in the present Flavouring Group Evaluation, for which the flavour industry has provided use and use levels (Flavour Industry, 2014). The mTAMDI value is only given for the highest reported normal use level.

Table C.5: Estimated intakes based on the mTAMDI approach

FL-no	EU Register name	mTAMDI (μg /person per day)	Structural class	Threshold of concern (μg /person per day)
16.119	N-(2-Methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide	520	Class III	90

mTAMDI: modified Theoretical Added Maximum Daily Intake.

Appendix D – Metabolism

D.1. Absorption, distribution and elimination

A single oral dose of 50 mg/kg of [^{14}C]-*N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide (^{14}C incorporated on the carbonyl group) (Figure D.1) was administered to male and female Sprague–Dawley rats and the tissue distribution of radioactivity was investigated using the technique of whole-body autoradiography. Pairs of rats (one male and one female) were killed at 1, 3, 9, 24 and 72 h post-administration and sagittal sections were obtained at six levels through the carcasses, freeze-dried and exposed to phosphor-imaging plates for 3 days. The distribution of radioactivity was assessed qualitatively, by visual inspection of the images. Following oral administration, radioactivity (representing *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide and/or its metabolites) was absorbed and widely distributed into all tissues throughout the animal body. Tissue radioactivity concentrations were greatest during 1–3 h after dosing, with the majority of tissues containing concentrations similar to that observed in the blood. The greatest concentrations were associated with the excretory organs (liver, kidney, gastrointestinal tract and urinary bladder). The lowest concentrations of radioactivity occurred in the lens of the eye. By 72 h after dosing (the final sacrifice time), radioactivity concentrations had declined but were still present (at low levels) in the gastrointestinal tract, liver, kidney, lung, blood, skin, tongue, and the harderian and lacrimal glands. Apart from those tissues which are gender specific, there were no sex-related differences in the distribution of [^{14}C]-*N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide and/or its metabolites (Penketh, 2009).

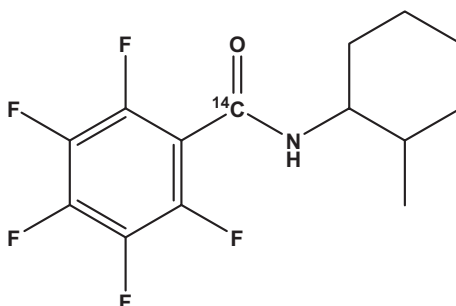


Figure D.1: The radiolabeled [^{14}C]-*N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide

D.2. Metabolism

In vitro metabolism study in rat liver microsomes

In an *in vitro* metabolism study, 1, 3, 30 and 100 μM of [^{14}C]-*N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide were incubated (at 37°C) with rat liver microsomes (0.5 mg/mL) for 0, 10, 30, 60 and 120 min. At the end of the required incubation time, the reactions were stopped by addition of acetonitrile. The samples were centrifuged and the supernatants were submitted for analysis. Analysis of the treated samples by high-performance liquid chromatography (HPLC) showed that after 120 min of incubation [^{14}C]-*N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide was almost completely metabolised to form up to 16 different metabolites (Kirby, 2009).

In a mass spectrometry (MS) analysis of the 30 μM and 100 μM samples (120 min incubation), only hydroxylated metabolites of *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide were identified. In all cases, it was the methyl cyclohexyl ring that underwent the hydroxylation step (see Figure D.2). Hydroxylation did not occur at the nitrogen or at C1 in the cyclohexyl ring (Kirby, 2009).

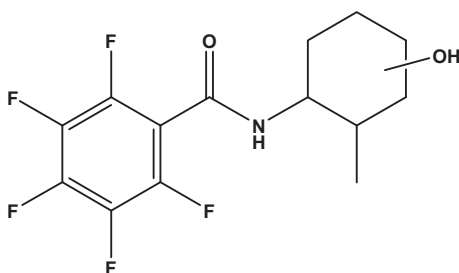


Figure D.2: Interpretation of metabolite structure based on MS and chemical considerations

In vitro metabolism study in isolated rat hepatocytes

An *in vitro* study was conducted at Xenotech LLC Labs in 2015 (IFF, 2016) to determine the metabolic stability of *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119] using a suspension culture of cryopreserved rat hepatocytes. After 0, 15, 30, 60, 90 and 180 min of incubation (1 μ M of substrate, 1 million cells per mL), the reactions were stopped by the addition of stop reagent (i.e. acetonitrile containing an internal standard). Additional incubations were carried out with the hepatocytes by replacing the test article with 7-ethoxycoumarin (positive control substrate) to confirm metabolic competency of the cells. The samples were centrifuged and the supernatant fractions analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Analysis was determined for the test article in each incubation solution. Results were determined by comparison of the analyte/internal standard (IS) peak-area ratios over the time course of the experiment and expressed as ‘% Parent Remaining’. Zero-time incubations served as the ‘100%-remaining’ values. *In vitro* half-life (min) and *in vitro* intrinsic clearance values (μ L/min per 10^6 cells) were estimated.

The hepatocyte cell viability ranged from ~ 70% to ~ 51% over the course of the 3-h incubation and was determined to be within acceptable limits for this assay. The positive control data confirmed the metabolic competency of the rat hepatocyte test system. Under the conditions of this test system, *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide was rapidly cleared (3 h) by the cultured hepatocytes with an estimated half-life and *in vitro* clearance rate for the parent test article to be 18 min and 39 μ L/min per million cells, respectively.

The above described results indicate that the test material is rapidly metabolised in the liver.

D.3. Summary and conclusion

The kinetics of the candidate substance have been investigated in microsomes, in hepatocytes and *in vivo* by autoradiography (14 C incorporated on the carbonyl group) after oral administration to rats.

Results from the *in vitro* study on metabolism in rat liver microsomes show that *N*-(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide is readily hydroxylated by liver microsomes *in vitro*. These metabolites may be anticipated to be easily conjugated and excreted.

Also in hepatocyte incubations, rapid metabolism of the candidate flavouring substance was observed but no attempt was made to identify the biotransformation products. Next to hydroxylation, hydrolysis of the amide bond might occur which would result in formation of 2-methyl-cyclohexylamine and pentafluorobenzoic acid. However, there are no data to support this. In addition, there is no information whether defluorination *in vivo* might occur but from the molecular structure this seems unlikely.

Appendix E – Toxicity

Table E.1: Genotoxicity (*in vitro*)

Chemical name [FL-no]	Test system	Test object	Concentration	Result	Reference	Comments
<i>N</i> -(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119]	Ames test	<i>S. Typhimurium</i> TA98; TA100; TA1535; TA1537	500, 750, 1,000, 2,000 and 4,000 µg/plate	Negative ^(a)	Kirby (2008)	Valid
	Ames test	<i>E. coli</i> WP2 <i>uvrA</i>	500, 750, 1,000, 2,000 and 4,000 µg/plate	Negative ^(a)	Kirby (2008)	Valid

(a): With and without metabolic activation.

Table E.2: Genotoxicity (*in vivo*)

Chemical name [FL-no]	Test system	Test object	Dose	Result	Reference	Comments
<i>N</i> -(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119]	Micronucleus test	Mouse bone marrow cells	500, 1,000 and 2,000 mg/kg bw	Negative	Song (2008)	Valid

bw: body weight.

Table E.3: Toxicity studies

Chemical name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw per day)	Reference	Comments
<i>N</i> -(2-methylcyclohexyl)-2,3,4,5,6-pentafluoro-benzamide [FL-no: 16.119]	Rats, Wistar Han: HsdRccHan:Wist strain 5 male and 5 female/group	Feeding	0, 750, 1,500 and 3,000 mg/kg in feed corresponding to 67, 130 and 262 mg/kg bw per day	28 days	Study not suitable for derivation of a NOAEL	Dunster (2009)	Inhibition of movement was seen in all dosage groups
	Rats, Crl: Wistar Han IGS rats 10 male and 10 female/group	Feeding	0, 750, 1,500 and 3,000 mg/kg in feed corresponding to 0, 55, 108 and 218 mg/kg bw per day for males and 0, 66, 125 and 255 mg/kg bw per day for females.	90 days	55	Bauter (2013)	

bw: body weight.